EFFECTS OF 3D MICROENVIRONMENTS ON DRUG EFFICACY TO BREAST CANCER CELL

by
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Abstract

Cancer cell is the type of cell that is not regulated by the normal cell checkpoints. It shows an aberrant cell proliferation and a high metastatic potential. Caner is one of the leading causes in human death globally among all diseases. There are more than 100 kinds of cancer types, including breast, lung, colorectal, prostate, etc. Currently, surgical method, radiation therapy, targeted therapy and chemotherapy have been widely adopted to treat cancers depending on the cancer type and their statuses. In breast cancer, there are four main types: luminal A, B, triple positive and triple negative based on the receptor: estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor (HER2) status. Among these four subtypes, triple-negative breast cancer (TNBC) has the lowest 5-year survival rate and highest recurrence rate[1]. Currently, there are four non-surgical therapies: target therapy, hormonal therapy, immunotherapy, and chemotherapy. However, due to the fact that TNBC lacks all three types of receptors, hormonal and target therapy are not the options for treating TNBCs. As of 2021, there are only two immunotherapeutic drugs approved in treating TNBC, which have to be used in combination with other chemotherapeutic drugs[2,3]. Therefore, chemotherapy remains the only non-surgical therapy in treating TNBC.

Anticancer drugs can be (1) cytotoxic (alkylating agent and anti-metabolite), (2) inhibit cell growth (mitotic inhibitor) or (3) disrupt enzyme functions (PARP inhibitor or EGFR inhibitors). Even though it has been proved that 3D models better mimics *in vivo* conditions, 2D monolayer assay is still used to learn drug efficacies[4]. Partly due to the

reason for its reproducibility. In addition to that, and many estimators can be calculated, i.e. IC_{50} values, maximal inhibition, etc. However, these estimators are usually calculated based on the inhibition curve, which can potentially be confounded by the variability of cell proliferation rate, cell density and doubling time. Consequently, the mechanism of anticancer drugs can be misleading.

In this project, we are introducing a high-throughput method to learn drug efficacies in both 2D and 3D by utilizing H33342 and PI stains for live and dead, respectively. We also showed how IC_{50} was confounded by the growth rate and therefore, growth rate inhibition should be used in combination to learn drug responses, which potentially decouples the effect of different proliferation rate in 2D and 3D. In this study, we studied the drug responses of 6 drugs on MDA-MB-231 and MCF-7. From the preliminary results, it was shown that during the first 48- and 72-hour treatment, none of the drugs were able to kill MDA-MB-231 compared to the initial cell count. However, a decrease in the live cell count was observed from 48-hour to 72-hour treatment, implying a longer period observation time might be required to further understand the mechanism. In addition to that, MDA-MB-231 responds to all drugs the same in both 2D and 3D. However, on the contrary, MCF-7 showed different drug responses in 2D and 3D.

In conclusion, we used a high-throughput method to screen 6 drug responses in both 2D and 3D. For MDA-MB-231, the drug responses in 2D and 3D were the same while in the case of MCF-7, it showed different responses in 2D and 3D. Therefore, to screen a chemotherapeutic drug in 3D or not seems in a cell-line dependent manner. For our preliminary conclusion, it seems TNBC cells (MDA-MB-231) response the same in both

2D and 3D while non-TNBC (MCF-7) responds differently. However, to confirm the trend, more repeat data should be collected, and more cell lines should be tested.

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1 Introduction

1.1 Background

Breast cancer is one of the leading causes of cancer-related death in the United States. It is estimated that 1/8 females (12.5%) will develop breast cancer in their lifetimes[5]. There are four stages in breast cancer: the earliest stage is stage 0 (carcinoma *in* situ) and ranges from stage I through IV, which stage IV is metastatic and is the most malignant and aggressive stage of the diseases. Early detection helps breast cancer patients to increase the 5-year survival rate from 75% to 90%[1,5]. Even though early stage breast cancer can be highly curable, but the 5-year survival rate for metastatic breast cancer patients is only 20%[6]. Breast cancer is a heterogeneous disease with multiple subtypes, which is characterized by the receptor status, including estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor (HER2). Triple-negative breast cancer (TNBC) is the subtype that lacks all three types of receptors, and among all subtypes, is the most malignant with poorest prognosis clinically, which is accountable for 15%-20% of breast cancer patients[7,8]. Some of common breast cancer cell lines with detailed receptor status are listed in **Table 1.** Moreover, unfortunately, the 5-year diseases-free survival rate were 57.5% for TNBC patients and 75.3% for Non-TNBC patients[9]. While there are various treatments for breast cancer, selection of treatment mainly depends on the types of the breast cancer. Current therapeutic strategies include surgery, radiotherapy, target therapy, hormonal therapy, immunotherapy, and chemotherapy.

Subtype	Receptor Status	Cell Line Examples
Luminal A	ER+, PR+, HER2-	MCF7
Luminal B (HER2+)	ER+, PR+, HER2+	BT474
HER2 positive	ER-, PR-, HER2+	HCC1569
Basal Like	Triple negative	MDA-MB-231

Table 1. 1 Common subtypes of breast cancer cell lines and corresponding receptor status

1.2 Anticancer Treatments

Surgical and radiotherapeutic methods are commonly used to treat various types of cancers, including breast cancer, to remove, destroy, shrink, and contain the growth of tumor growth locally. Target and hormonal therapies are used to treat non-TNBC cancer, which expresses one of the three receptors. Current target and hormonal includes trastuzumab(Herceptin), tamoxifen (Saltamox) and aromatase inhibitor, such as anastrozole (Arimidex), exemestane(Aromasin) and letrozole (Femara), etc. Immunotherapy is new treatment method that is yet to be explored. As of 2021, there are only two immunotherapeutic drugs approved for breast cancer treatment, including atezolizumab and pembrolizumab[2,3]. Even though atezolizumab and pembrolizumab showed efficacy in treating TNBCs, these drugs are still suggested to be used in combination with other chemotherapeutic drugs. Therefore, to treat TNBC patients, chemotherapeutic drugs are still the most frequently used in clinical[8]. Chemo-drugs are usually taken orally or intravenously/intramuscularly, depending on the type and the stage of the cancer. Chemotherapeutic drugs can destroy and/or control cancer cells through

the body depending on the type. Chemotherapeutic drugs can be further divided into five major categories, including alkylating agents, anti-metabolite, mitotic inhibitors, poly ADP-ribose polymerase (PARP) inhibitors and epidermal growth factor receptor (EGFR) inhibitors.

1.3 Chemotherapeutic Drugs

Alkylating agents were the first non-hormonal compounds to be active against malignant cells, including leukemia, lymphoma and breast cancer[10]. Alkylating drugs are believed to exert cytotoxic effects through covalent linkage of alkyl group to DNA[10]. Thus, prevent cell division. Anti-metabolite drugs are also cytotoxic to breast cancer cells, in which they work by mimicking a false building block in DNA and RNA synthesis[11]. With the drugs incorporated into the DNA, cells are believed to stop replicating, thus induce apoptosis or cell cycle arrest depending on the drugs. Since cancer cells are characterized by their uncontrollable ability to grow, divide and spread at the developed or distant sites. Mitotic inhibitors are the drugs targeting cancer cell arrest during the mitosis by disrupting microtubules[12]. In addition to these clinically approved drugs in treating TNBC patients, more chemotherapeutic drugs that aiming certain over-expressed targets are currently under experimental/clinical evaluation. PARP and EGFR inhibitors are novel drugs to treat several kinds of cancer cells, including non-small cell lung cancer cell, breast cancer, etc. PARP is a multifunctional enzyme, upon activation, plays a role in pathogenesis of various cardiovascular and inflammatory diseases. At the same time, it is also known for its ability to repair single strand damaged DNA[13,14]. In TNBCs, EGFR is usually overexpressed and thus becomes a potential therapeutic target. There are two types of

EGFR inhibitors, small molecular tyrosine kinase inhibitor (TKI) and monoclonal antibody (mAb) for treatment of cancers, including non-small cell lung cancer, colorectal cancer and breast cancer[6]. **Table 2.** summarizes the current clinically approved chemotherapeutic drugs in treating TNBCs.

Therapy Type	Regimen	Brand	Agent
Hormonal Therapy	Endocrine Therapy	Nolvadex	Tamoxifen
	Aromatase Inhibitor	Arimidex Femara	Anastrozole Letrozole
Target Therapy	Antineoplastic Agent	Herceptin	trastuzumab
Chemotherapy	Alkylating Agent[10]	Thioplex	Thiotepa
	Anti-Metabolite[11]	Trexall Adrucil	Methotrexate 5-Flourouracil (5-FU)
	Mitotic Inhibitor[12]	Taxol Velban	Taxol Vinblastine Sulfate
	PARP Inhibitor[13,14]	Lynparza	Olaparib
	EGFR Inhibitor[6]	N/A	Under development

Table 1. 2 Current approved/under development therapeutic drugs in treatment of breast cancer

1.4 In vitro Microenvironments and Quantification

The development and utilization of models that physiologically recapitulate *in vivo* condition is emerging and is believed to be essential in understanding the pathophysiology of tumor cells, and to discover new anti-cancer drugs. As a result, models that mimic the *in vivo* tumor microenvironment are developed, including spheroids and organoids[4,15,16]. 3D model is able to mimic cell-cell interaction, cell-ECM interaction, oxygen and nutrient gradients, variable stiffness and cell polarity, which are all lacked in 2D monolayer culture[15,17–20]. Such characteristics of 3D model better mimics the microenvironment where cells reside in tissues[16]. Thus, these models can bridge the gap between *in* vitro 2D cultures and expensive *in vivo* animal studies. However, even though 3D spheroids/organoids better mimics *in vivo* conditions, current methods are bulk measurements. Up to date, most cytotoxicity assays are indirect measurement (MTT/HTT assay, BrdU assay and cellular ATP levels), which lacks validation and requires normalizations.

1.5 IC_{50} as an Indicator of Drug Responses

As aforementioned, 3D is believed to bridge the gap between *in vitro* and *in vivo*. Prior to clinical trials, drugs are screened both *in vitro* and *in vivo*. Inhibition curve is used to study the effects of drug-treated conditions relative to drug-free conditions. From the inhibition curve, several estimators can be calculated to study the drug efficacy, which are IC_{50} (half maximal inhibitory concentration): measures the concertation of a substance is needed to inhibit 50% of a given biological process; and E_{max} (maximal inhibition), which measures the maximal ability of a drug to inhibit cell growth. The advantage of those

parameters is that both IC_{50} and E_{max} can estimate the efficacy of various drugs within a system. However, IC_{50} and E_{max} are easily being impacted by the experimental conditions, therefore comparing IC_{50} and E_{max} in different experiment and setups is more challenging.

1.6 Project Overview

In the present study, we introduce a high-throughput fluorescent imaging system that was previously developed by our lab to monitor drug treatment on MDA-MB-231 and MCF-7 in both 2D and 3D cell cultures on a single-cell level. 6 anti-TNBC chemotherapeutic drugs were selected with various concentration (0.1 mM to 1 nM). Among the chosen drugs, there were 2 FDA unapproved drugs and 4 FDA approved drugs. Both cell lines were exposed to drugs for 48 hour and 72 hours to learn the mechanism of the drugs. To take the doubling time into account, the live, and dead cell count 0 hour prior to treatment were recorded by utilizing Hoechst 33342 nucleic stain(H33342) and propidium iodide (PI) stain.

After the information collected, the drug-response curve is then plotted to learn the drug efficacy in treating MDA-MB-231, which is a TNBC cell line and MCF-7, which is a non-TNBC. The IC_{50} and E_{max} were calculated to further understand the potency of drugs. As the IC_{50} and E_{max} can be easily confounded by the variability cell proliferation rate, we also graphed growth rate inhibition, which we used to compare the 48-hour and 72-hour treatment to 0-hour treatment.

Given the multiplex advantage of our algorithm, we further learned the cell viability (live cell/ total cells) in each condition and then compared the viability under different drugs, concentration, micro-environment, treatment time and cell lines.

2 Material and Methods

2.1 Materials

2.1.1 Compounds and reagents

The drugs were obtained from *Selleckchem*. The collection includes 10 drugs, all maintained at 10mM in DMSO. Hoechst 33342 was purchased from Invitrogen and Propidium Iodide(PI) was purchased from ThermoFisher.

Reagent	Source
Corning® Collagen I, High Concentration, Rat Tail	CORNING
Hoechst 33342 (H33342)	Thermo Fisher Scientific
Propidium Iodide (PI)	Thermo Fisher Scientific

Table 2. 1 Reagents used in this study

Paclitaxel (Taxol)	Selleckchem
Fluorouracil (5-FU)	Selleckchem
Vinblastine Sulfate	Selleckchem
Thiotepa	Selleckchem
Methotrexate	Selleckchem
Afatinib	Selleckchem
Iniparib	Selleckchem

Table 2. 2 Drugs used in this study

2.1.2 Cells

MDA-MB-231 and MCF-7 were obtained from the ATCC. Both cells were cultured in DMEM with 1% (v/v) Penicillin-Streptomycin and 10% (v/v) FBS.

Cell line	Subtype [19]	Source
MCF7	Luminal A	American Type Culture Collection
MDA-MB-231	Triple negative	American Type Culture Collection

Table 2. 3 Cell lines used in our drug response testing experiment

2.1.3 Cell culture in 2D and 3D

MCF-7 and MDA-MB-231 were maintained as monolayer cultures in 10-cm dishes in the media described above in an incubator at 37 °C. Prior to preparing cell cultures in 2D and 3D, cells were trypsinized and collected the supernatant for cell counting. To count the cell density, 10 µL of cell solution was stained 30 µL stain (1%(v/v) Hoechst and 1%(v/v) PI) in triplicates. For a better counting result, the plate was shaken with 300 rpm for 5 minutes and then leave on the shaker for 20 minutes at the room temperature before 15-minute incubation.

To prepare 3D cultures for high-throughput screening, cells were seeded at 2000 cells/well in DMEM (as described above) and 2.5 mg/ml Collagen type I , which was diluted from the reagent mentioned above, in 96-well round-bottom black-walled plates (Corning).

For 2D high-throughput screening, cells were cultured in the same media, same cell concentration and the same kind of 96-well plate. Cell viability was checked using the same stain as described above.

2.1.4 Cytotoxicity assay

All drugs were pre-diluted to 10mM solution in DMSO or DI water (based on the manufacture's recommendation) as solvent stored at -80 °C. Cells were plated as described for 2D or 3D culture, and then treated with compound at the concentration mentioned above for 48 or 72 hours. To prepare the final concentration, compounds were serial diluted into the final concentration (0.01 mM to 1 nM). To quantify cell viability, cells were stained as described above for 1-hour incubation. Due to the diffusivity, 25 µL and 50 µL was added to each well in 2D and 3D culture respectively.

2.2 Image processing

2.2.1 Immunofluorescent analysis of 2D and 3D cultures.

Both 2D and 3D were stained the same way (H33342 and PI). After stained, the plate was imaged under two fluorescent channels (DAPI and TRITC) using a 2X objective, which gives a full view of each well.

2.2.2 Cell Counting and Algorithm

After the images were obtained, a detection algorithm was used to identify the objects based on the three properties, including H33342 intensity, PI intensity and cell morphology. This algorithm is a novel way of tracking cells and eliminate the duplicated detections under different channels. Each object's H33342 intensities, PI intensities,

positions in cartesian coordinates, distance from the bottom and the radius of the signals to the center of the well was recorded for further analysis. To get accurate cell counting and further eliminate the noises, a gating process was employed to assure the quality of the data. All codes were written as MATLAB script.

3 Results

3.1 H33342 vs PI intensity map shows consistent two population to separate live and dead cells

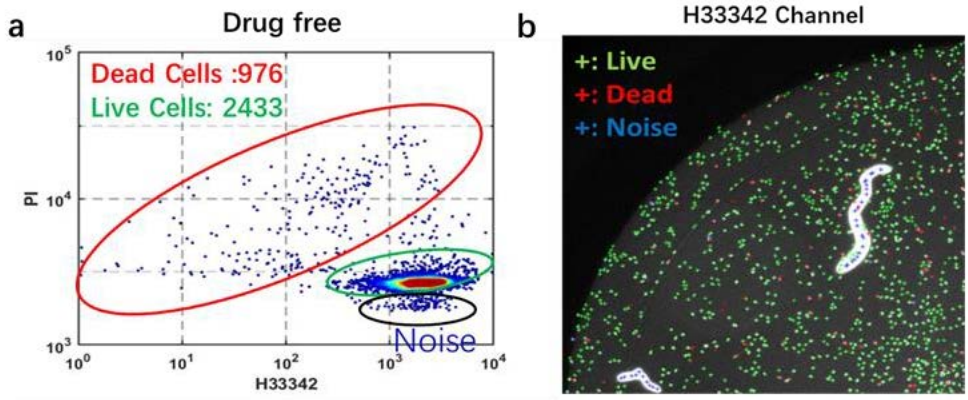

Figure 3. 1 Gating map for live and dead index (LDI) cell counting. (a) H33342 vs PI intensity map; (b) The corresponding overlapping validation image.

Figure 3.1(a) is a typical gating map that shows the live and dead cell count (LDI) within each well. Since H33342 is a nucleic stain, which can label all cells, regardless of live or dead. Therefore all objects have some extent of H33342 intensity. However, PI is a huge molecule that is only permeable to dead cells, therefore, only dead cells have PI intensity. As shown in Figure 3.1 (a), there are two major populations: green for live cells and red for dead cells. Each time, the live and dead cells are selected in different polygons that is defined by the user. All the points that are neither in the green nor the red circle will be

recognized as noise, which will not be counted when outputted. Figure 3.1(b) shows the corresponding validation image. The green dots are the points in the green circle. Given the (x,y) coordinates of each point, the overlapping image, therefore, is created for visual validation. As shown in Figure 3.1(b), the bulk mass is successfully recognized as noises and eliminated. Therefore, the gating method is used to analyze drug treatment data.

3.2 Taxol treatment over MDA-MB-231 and MCF-7 in 2D and 3D

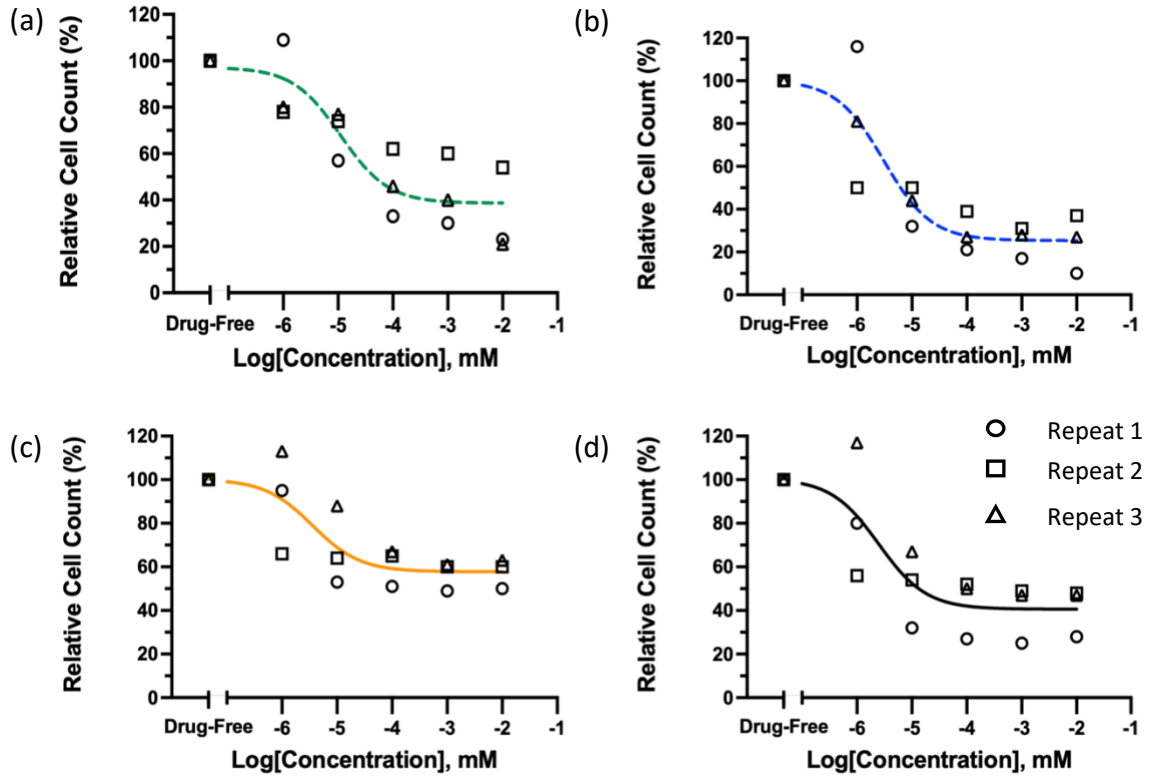

Figure 3. 2 Inhibition curve of Taxol treatment on MDA-MB-231. The line in each plot is the average line of the three repeats. Taxol treatment on MDA-MB-231 in 2D for 48hr; (b) Taxol treatment on MDA-MB-231 in 2D for 72hr; (c) Taxol treatment on MDA-MB-231 in 3D for 48hr; and (d) Taxol treatment on MDA-MB-231 in 3D for 72hr.

Taxol is a chemotherapeutic drug that has various application in treating cancers, including breast cancer, pancreatic cancer, ovarian cancer, etc[8]. Taxol is a microtubule

inhibitor, which functions by disrupting microtubules and thus interferes mitosis[12]. For each concentration, the live cell count is calculated from the GUI mentioned in the earlier section. To get the inhibition curve (relative cell count), the cell count in treated conditions is normalized by the drug-free condition. When MDA-MB-231 is treated by taxol for 48hr, both 2D and 3D showed similar average line as shown in Figure 3.2 (a) and (c). However, from Figure 3.2(a) and (c), even though the trend of the average line is similar in 2D and 3D, their maximal inhibition rate is different, i.e. E_{max} (2D) = 67.3% and E_{max} (3D) =39%. For 72-hour treatment data, similar trend between 2D and 3D is observed, as shown in Figure 3.2 (b) and (d), respectively. The E_{max} (2D) = 72% and E_{max} (3D) =60%. When compare the 48-hour and 72-hour within the same microenvironment (2D and 3D), 72-hour treatment showed better maximal inhibition as the culture exposed to drug for longer time.

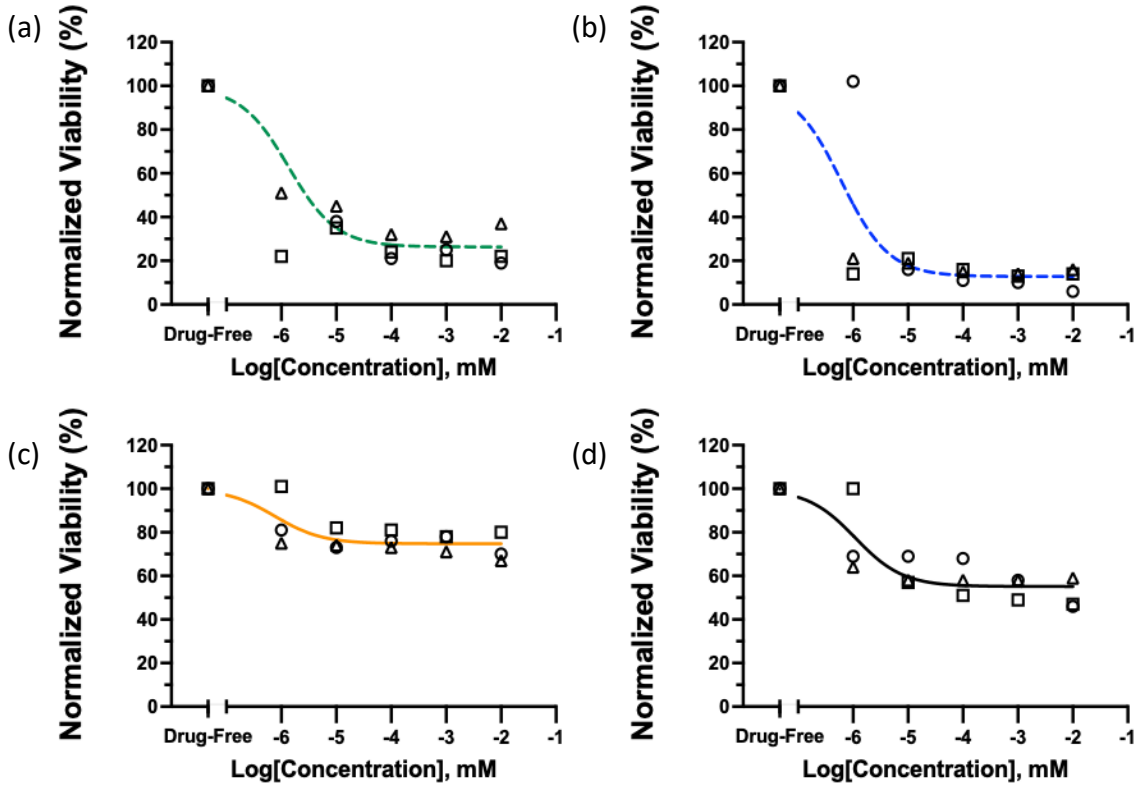

Figure 3. 3 Inhibition curve of Taxol treatment on MCF-7. The line in each plot is the average line of the three repeats. (a)Taxol treatment on MCF-7in 2D for 48hr; (b) Taxol treatment on MCF-7in 2D for 72hr; (c) Taxol treatment on MCF-7 in 3D for 48hr; and (d) Taxol treatment on MCF-7in 3D for 72hr.

Taxol treatment over MCF-7 is shown in Figure 3.3. Similar to MDA-MB-231 treatment, at both treatment time points, MCF-7 response to Taxol is very similar. However, a big difference in E_{max} value is observed between 2D and 3D. The E_{max} for 48hr-treatment 2D and 3D is 74.0% and 27.7%, for 72hr-treatment 2D and 3D is 88.0% and 49.3% respectively. As shown in Figure 3.4, there was a significant difference between 2D and 3D in E_{max} in both MDA-MB-231 and MCF-7. Besides the $E_{max,}$ another commonly used estimator IC50 is also calculated based on the inhibition curve.

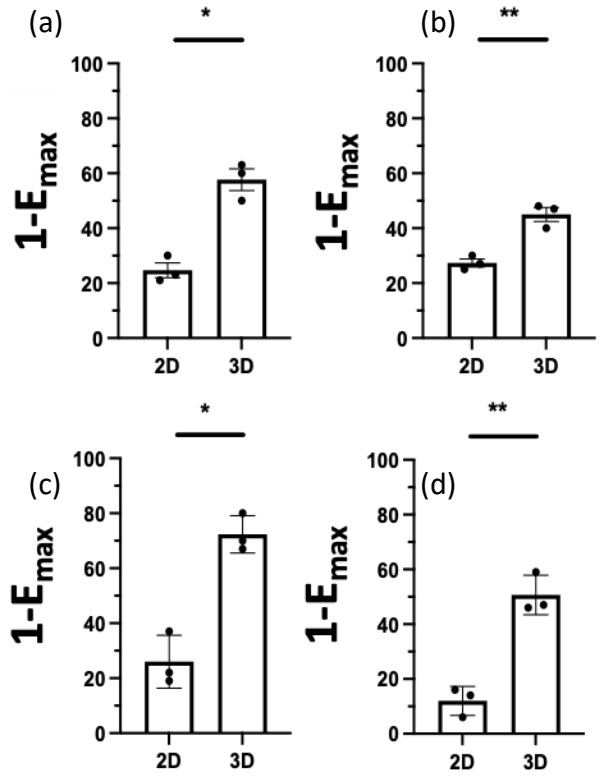

Figure 3. 4 Maximal inhibition of Taxol treatment over MDA-MB-231 and MCF-7. (a) Maximal inhibition of Taxol over MDA-MB-231 in 48hr; (b) Maximal inhibition of Taxol over MDA-MB-231 in 72hr; (c) Maximal inhibition of Taxol over MCF-7 in 48hr; (d) Maximal inhibition of Taxol over MCF-7 in 72hr; Students' t-test is used to calculate the statistics and sample size(N) = 3 for each of the bar plot. The value is mean ± SEM.

3.3 IC50 value as an indicator of drug potency

Half maximal inhibitory concentration (IC_{50}) is a measurement of the potency of a substance in inhibiting a specific biological or biochemical function. *In vitro*, IC_{50} measures the concentration of a substance (i.e., drug, inhibitors, etc.) is needed to inhibit 50% of a give biological process.

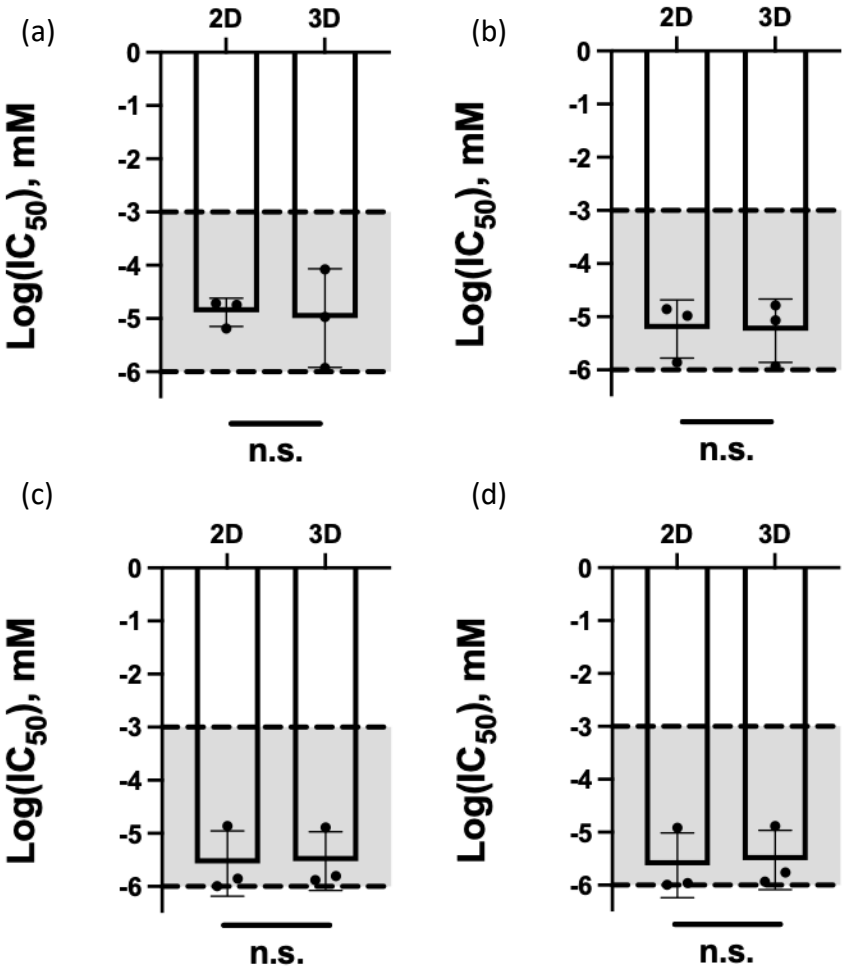

Figure 3. 5 IC_{50} of Taxol treatment over MDA-MB-231 and MCF-7. (a) IC_{50} of Taxol over MDA-MB-231 in 48hr; (b) IC_{50} of Taxol over MDA-MB-231 in 72hr; (c) IC_{50} of Taxol over MCF-7 in 48hr; (d) IC_{50} of Taxol over MCF-7 in 72hr; Students' t-test is used to calculate the statistics and sample size(N) = 3 for each of the bar plot. The value is mean ± SEM.

As shown in Figure 3.4 (a) and (b), the IC_{50} values of Taxol over MDA-MB-231 does not show any difference between 2D and 3D in both 48-hour and 72-hour treatment. Figure 3.4 (c) and (d) shows the IC_{50} value of Taxol over MCF-7, similar to the MDA-MB-231, there's no statistical difference between 2D and 3D. However, as aforementioned, the E_{max} between 2D and 3D under each treatment condition is different, which showed a

statistical different in the values in MDA-MB-231 and in MCF-7. Table 3.1 summarizes the IC_{50} and E_{max} values in each condition.

Cell line	Treatment Time(hr)	2D		3D	
		IC_{50} (nM)	E_{max} (%)	IC_{50} (nM)	E_{max} (%)
MDA-MB-231	48	14.7	67.3	32.1	59%
	72	5.9	75.3	5.5	42.5
MCF-7	48	5.4	74.0	5.3	27.7
	72	2.4	88.0	3.0	49.3

Table 3. 1 Summary of the discrepancy between IC_{50} and E_{max} between 2D and 3D

3.4 Growth rate measures the drug response and corrects the confounders that caused by cell proliferation rate.

It has been previously shown that the IC_{50} and E_{max} values can be confounded by the variability in the cell proliferation rate, which can be caused by the cell density, media composition and the biological difference in cell doubling time. Therefore, it was suggested that using the growth rate to evaluate the drug response potentially corrects these confounders and takes cell proliferation effects into account[21].

Cell line	Log (Concentration) (mM)	48hr		72hr	
		2D	3D	2D	3D
MDA-MB-231	-1	2400±27	2000±66	2100±230	1900±44
	Drug-free	6400±1000	3500±180	9400±2000	4800±1000
MCF-7	-1	3500±203	3100±390	3200±263	2600±76
	Drug-free	14000±1900	4300±610	31000±6200	5200±260

Table 3. 2 Cell Count in each treatment conditions. Values are Mean ± SEM

Table 3.2 summarizes the cell count in each condition. In the highest dosage treatment condition, the cell counts in 2D and 3D are similar in both cell lines. Given that they were seeded at the same cell count initially, the efficacy in inhibiting cell growth from time 0 to the time t should be the same. However, as the inhibition curve is normalized by the drug-free conditions and there's a different growth rate, the efficacy appears to be different. As shown in Figure 3.3, Taxol seems more effective in inhibiting both cell lines in 2D and 3D, however, such effects could be confounded by the variability in the rate of cell proliferation. Therefore, the inhibition curve can lead to ambiguous results.

3.5 Growth rate measures the drug response and corrects the confounders that caused by cell proliferation rate.

As aforementioned, there is a difference in the cell proliferation rate in 2D and 3D and the inhibition curve could be hugely impacted by the variability in cell proliferation rates, which is caused by cell density, media composition and cell doubling time[21]. Therefore, a new factor that can decouple these effects is needed. Growth inhibition rate measures the proliferation rate from pre-treatment (the 0 hour treatment) to certain treatment time can potentially solve the problem. Here, the growth rate inhibition (GRI) is defined as $\frac{N_t}{N_0} - 1$, where N_t is the live cell count at time t post-treatment and N_0 is the live cell count at time 0 post-treatment. By using the method, the inhibition can be divided into three categories: if 1< GRI <$GRI_{drug\text{-}free}$, then it's partial inhibition; if GRI =0, then it's total inhibition and if GRI <0, it induces cell death. By using the method, the effects of growth rate can be decoupled, and the mechanism of the drug can be better understood in a single plot.

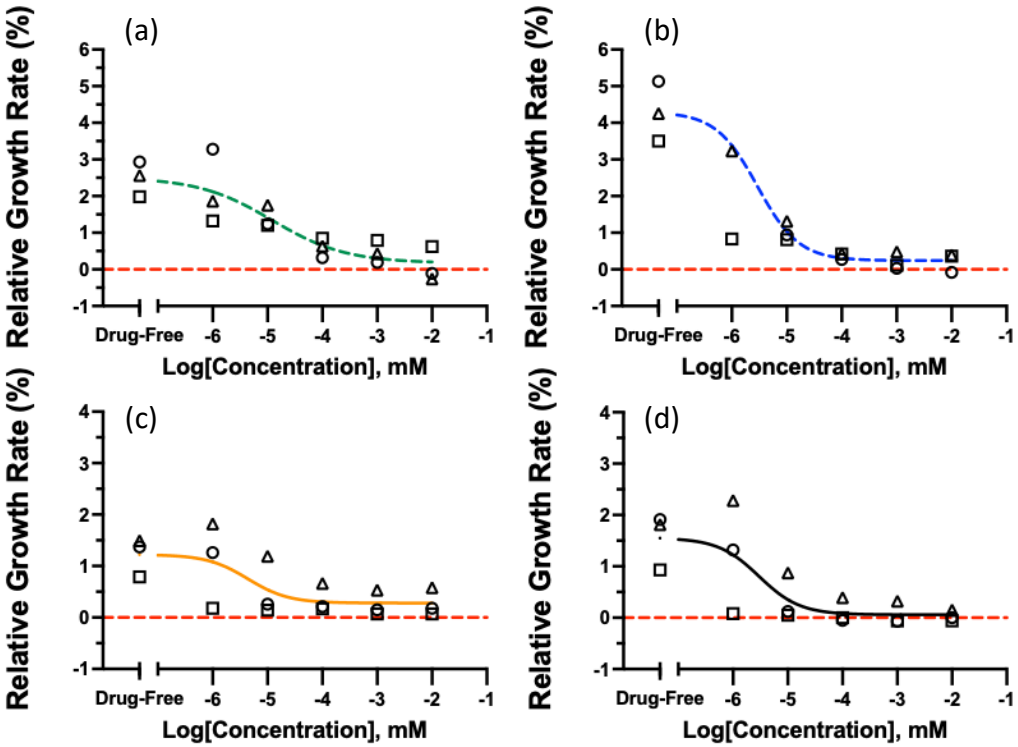

Figure 3. 6 Growth inhibition curve of Taxol treatment on MDA-MB-231; (a) Taxol treatment on MDA-MB-231 in 2D for 48hr; (b) Taxol treatment on MDA-MB-231 in 3D for 48hr; (c) Taxol treatment on MDA-MB-231 in 2D for 72hr; and (d) Taxol treatment on MDA-MB-231 in 3D for 72hr. The red dotted line is the pre-treatment cell count.

As shown in Figure 3.6 (a) and (b), the growth rate in drug-free in 2D and 3D differs hugely, however, the growth rate of highest Taxol concentration treated condition is very similar in 2D and 3D. Similar to that of 48-hour treatment, 72-hour treatment shows similar proliferation rate in higher Taxol treatment conditions between 2D and 3D. Interestingly, Taxol, as a mitotic inhibitor, is believed to prevent cell proliferation by disrupting microtubule formation and thus induce cell death. However, at all conditions, the growth rate is always a positive number, which means that Taxol only slows down the proliferation rate, but not fully inhibit the proliferation.

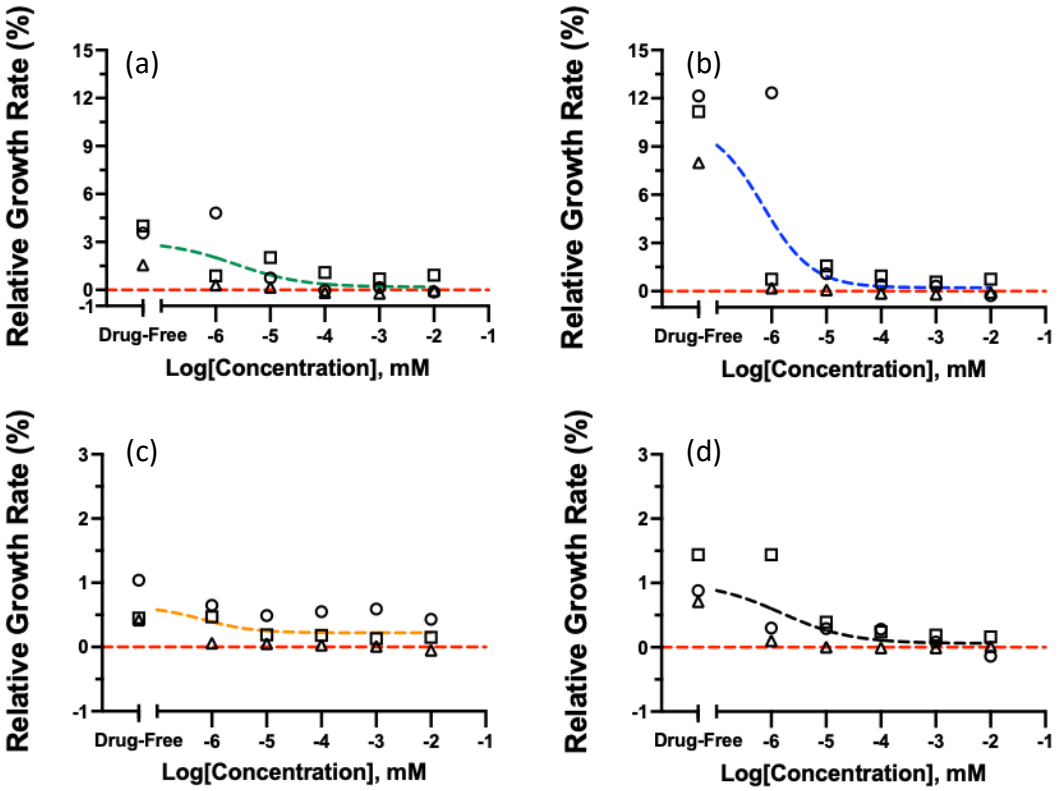

Figure 3. 7 Growth inhibition curve of Taxol treatment on MCF-7; (a) Taxol treatment on MDA-MB-231 in 2D for 48hr; (b) Taxol treatment on MDA-MB-231 in 3D for 48hr; (c) Taxol treatment on MDA-MB-231 in 2D for 72hr; and (d) Taxol treatment on MDA-MB-231 in 3D for 72hr. The red dotted line is the pre-treatment cell count.

Similar to the pattern in MDA-MB-231, MCF-7 also showed differences in the cell proliferation in 2D and 3D, but in a greater magnitude. Figure 3.7 (b) and (d) shows a huge difference in the cell proliferation in drug-free conditions, however, their relative growth rate at higher Taxol concentration shows a similar rate. Therefore, growth inhibition curve decouples the effects of the variability of proliferation in 2D and 3D.

3.6 Cell proliferation rate exceeds the Taxol inhibition effect in early stage

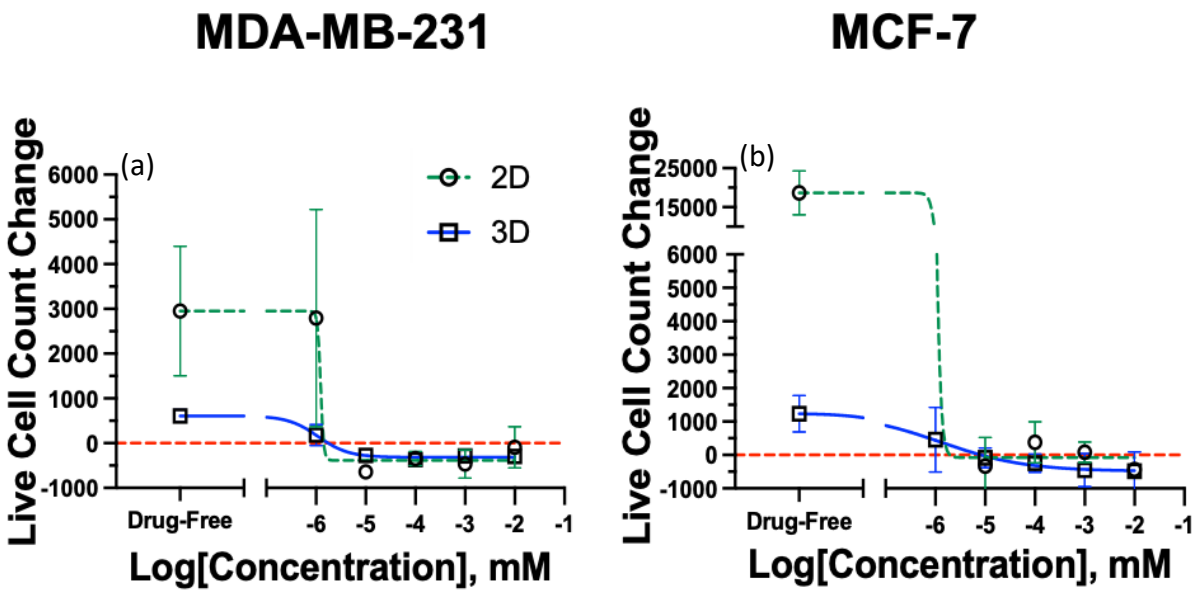

Figure 3.8 Live cell count change from 48-hour to 72-hour Taxol treatment under 2D and 3D; (a) MDA-MB-231 and (b) MCF-7

Figure 3.8 (a) shows a decrease in the MDA-MB-231 live cell count from 48-hour to 72-hour Taxol treatment in both 2D and 3D. The cell count change decreases except the lowest Taxol concentration (at Log[Concentration]=-6) and drug-free conditions, but as shown in Figure 3.6 (b) and (d), the cell count of 72-hour treatment is still higher than that of the 0-hour treatment, therefore, Taxol combats the MDA-MB-231 proliferation and within the first 72- hour treatment time, the cell proliferation rate exceeds the Taxol inhibition ability.

However, Figure 3.8 (b) shows a different pattern, MCF-7, when treated in 3D, shows a quite large decrease in the live cell count; however, in 2D, the live cell count change was in a marginal level, may underly a different Taxol treatment mechanism in 2D and 3D.

3.7 Using growth rate as an indicator to study drug treatment on MDA-MB-231 and MCF-7

Therapy	Regimen	Agent	Growth Rate at 10 μm (72hr Treatment)		Growth Rate at 10 μm (72hr Treatment)	
			2D	3D	2D	3D
Chemo-	Anti-Metabolite[11]	Methotrexate*	+	+	+	0
		5-Flourouracil (5-FU)*	+	+	+	0
	Mitotic Inhibitor[12]	Taxol*	+	+	0	-
		Vinblastine Sulfate*	+	+	0	-
	PARP Inhibitor[13,14]	Afatinib#	-	-	-	-
	EGFR Inhibitor[6]	Iniparib#	+	+	+	+

Table 3. 3 Summery of the growth rate of drug treatment on MDA-MB-231 and MCF-7; * stands for clinically approved drugs; # stands for clinically unapproved drugs; + means partial inhibition; 0 means total inhibition; and – means cell death

Table 3.3 summarizes the growth rate of different drug treatment effects on MDA-MB-231 and MCF-7. Surprisingly, regardless of the drug type, MDA-MB-231 showed similar growth rate inhibition throughout the treatment under 2D and 3D while MCF-7 showed micro-environment dependent responses. In addition to that, drugs that are believed to

be cytotoxic did now show much killing effects against MDA-MB-231, i.e. Methotrexate and 5-FU. Almost all drugs failed to induce MDA-MB-231 death, except afatinib, which failed clinically due to high cytotoxicity[22]. In the case of MCF-7, drug mechanism shows different responses in 2D and 3D, which might indicate drug treatment on MCF-7 is in an environment-dependent manner, but not MDA-MB-231.

4 Conclusions

In conclusion, from our drug responses data on MCF-7 and MDA-MB-231 cells, we obtained new insights about the difference of cell growth and death pattern in 2D and 3D *in vitro*. In addition, even though the IC_{50} values in 2D and 3D are very close to each other, their maximal inhibitory effects may var a lot as summarized in Table 3.2 under Taxol treatment. Moreover, the cell count at the maximum Taxol dosage were similar in different microenvironments in both MDA-MB-231 and MCF-7. However, due to the difference in the cell growth pattern in 2D and 3D, the inhibitory curve maybe confounded by the variability in the rates of cell proliferation, which is caused by the cell density, media composition and the different doubling time. Therefore, to use the growth rate curve as a substitute can potentially decouple the growth effects in different environments.

The growth rate graph gives more information than the inhibitory curve. For example, if the value is greater than 0, but less than the $GR_{drug\text{-}free}$, it means partial inhibition; if it equals 0, then it's total inhibition while induce cell death if it's less than 0. In addition to that, by using the single-cell-level analysis, we were able to learn when the cytotoxicity started to show effects in different cell lines.

Taxol, instead of killing cells, was able to prevent cell proliferation. It seems to have cytotoxic effects over both cell lines, but such effects only showed after cells were exposed to drug for 48 hours and was in a micro-environment independent for MDA-MB-231 and dependent for MCF-7.

We further tested the drug effects on 5 other drugs, which belonged to 4 different categories, including mitotic inhibitors, anti-metabolite, PARP inhibitor and EGFR inhibitors using the growth rate inhibition curve. We found that when MDA-MB-231 were treated with different drugs, 2D and 3D growth inhibition were very similar to each other while MCF-7 showed totally different drug responses in 2D and 3D.

In the future, to confirm the preliminary results, more biological repeats should be done on both cell lines. In addition, to learn the relation of the dependence of the drug treatment, more cell lines should be tested to confirm the general trend. Moreover, to better learn the how cells happen, more biomarkers should be added for a more comprehensive analysis.

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